LABEL-FREE BIOANALYTICAL METHODS FOR INVESTIGATING BIOMOLECULAR INTERACTIONS: A REVIEW

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Rena E. Ingram

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LABEL-FREE BIOANALYTICAL METHODS FOR INVESTIGATING BIOMOLECULAR INTERACTIONS: A REVIEW

Approved by:

Dr. M.G. Finn, Advisor
School of Chemistry and Biochemistry
Georgia Institute of Technology

Dr. Adegboyega Oylere
School of Chemistry and Biochemistry
Georgia Institute of Technology

Dr. Facundo Fernandez
School of Chemistry and Biochemistry
Georgia Institute of Technology

Dr. Eric Gaucher
School of Chemistry and Biochemistry
Georgia Institute of Technology

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Author

Rena E. Ingram

LIST OF FIGURES

LIST OF ABBREVIATIONS

DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
RI	Refractive Index
ITC	Isothermal Titration Calorimetry
30**S**	Svedberg Measurement Unit for Ribosomes
μM	Micromolar
nM	Nanomolar
SPR	Surface Plasmon Resonance
nm	Nanometer
nL	Nanoliter
CCD	Charged-Coupled Device
MetJ	E. coli Methionine Repressor
E. coli	*Escherichia coli*
SA	Streptavidin
CM5	Carboxymethylated 5 Sensor Chip
BSI	Backscattering Interferometry
HeNe	Helium-neon
FFT	Fast Fourier Transform
MeCN	Acetonitrile
GM1	Monosialotetrahexosylganglioside
pM	Picomolar

FAAH	Fatty Acid Amide Hydrolase
CXCR4	CXC Chemokine Receptor Type 4
$GABA_B$	Gamma-Aminobutyric Acid Type B
AChE	Acetylcholinesterase Inhibitors
fmol	Femtomole
pL	Picoliter
RSVN	Respiratory Syncytial Virus Nucleocapsid (Gene)
PfEBA175	*Plasmodium falciparum* Erythrocyte Binding Antigen 175
PfRH5	*P. falciparum* Reticulocyte Binding Protein Homologue 5
µL	Microliter
BLI	Bio-layer Interferometry
APOBEC3F	Apolipoprotein B MRNA Editing Enzyme Catalytic Subunit 3F
HIV-1	Human Immunodeficiency Virus Type 1
kDa	Kilodalton
mM	Millimolar
Da	Dalton
A431	Human Epidermoid Carcinoma Cells
SPRi	Surface Plasmon Resonance-based Imaging
PCSK9	Human Proprotein Convertase Substilisin Kexin Type 9
IL-12	Human Interleukin 12
FET	Field-Effect Transistor
CNT	Carbon Nanotubes
SiNW	Silicon Nanowires

GFET	Graphene Field-Effect Transistor
ME	Magnetoelastic

SUMMARY

Interactions between biological molecules such as DNA, RNA, protein, lipids, and carbohydrates are critical to the understanding of all biological, physical, and chemical processes such as protein function, disease diagnosis, and drug discovery. (Citartan, 2013) Most of the techniques currently used to detect and quantify biomolecular interactions are assisted by foreign molecules that are either permanently or temporarily attached to the molecule of interest; such labels most often fluorescence, luminescence, are radioactive, or are large enough to be easily detected (nanoparticles). (Syahir, 2015) Fluorescent labeling detection methods, the most common and convenient, are attractive due to their stability, easy manipulation, and high sensitivity and dynamic range. (Syahir, 2015) However, label-free and real-time detection methods are of high demand due to a number of potential disadvantages of the labels or the methods used to attach them: 1) altering the structure, conformation, or functional properties of the biomolecule of interest, 2) occupying the active site(s) of biomolecules thereby changing their binding affinity, 3) producing false-positive results by interacting with unanticipated components, 4) loss of sample during the labeling and purification process, 5) high sensitivity to changes in environmental conditions, and 6) potential to be tedious and expensive. (Citartan, 2013) (Sun, 2014) (Fechner, 2014) (Sang, 2015)

CHAPTER 1: LABEL-FREE TECHNIQUES

Label-free methods are used to track molecular events using molecular biophysical properties such as molecular weight, refractive index (RI), and molecular charge. (Syahir, 2015) Label-free technologies can be simple, low-cost, and able to monitor binding interactions without disturbing the native interaction of molecules. (Kussrow, 2011) The ability to use native biomolecules makes these methods especially suitable for biologically relevant approaches. (Nguyen, 2015) Here I discuss several of the most widely used label-free methods and highlight a few examples of each system.

1.1 Isothermal Titration Calorimetry

Isothermal Titration Calorimetry (ITC) is used to obtain the absolute thermodynamic parameters (affinity, stoichiometry, enthalpy, enthalpy, and Gibbs free energy) driving a molecular binding interaction of interest. (Duff, 2011) ITC (Figure 1) is a heat-flux calorimeter that functions by titrating one reactant into a second reactant under isothermal conditions and measures the amount of power over time (ucal/sec) required to maintain a constant temperature upon binding of the two reactants in comparison to a reference cell by heat released (exothermic reaction) or absorbed (endothermic reaction). A series of injections are performed over time and heat changes of a few millionths of a degree Celsius are induced if binding occurs. The experimental output is a plot of the power needed to maintain constant temperature as a function of time which is fitted using the Wiseman isotherm, $c = nK_a[\mathrm{R}]_t$. (Callies, 2016)

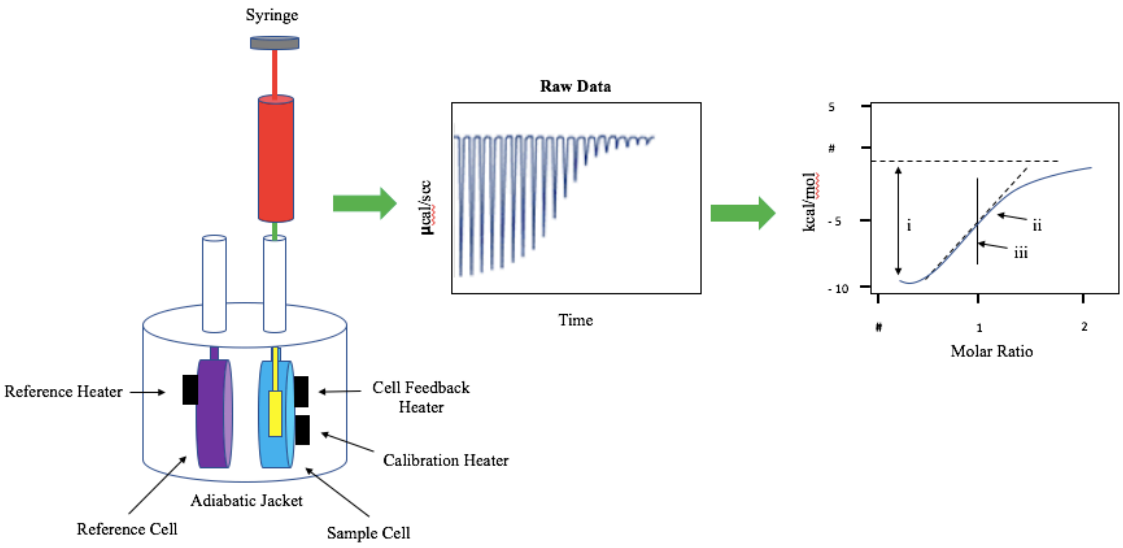

Figure 1. *Isothermal titration calorimetry schematic. The microcalorimeter includes a reference cell that contains water and another cell that contains one of the binding partners. Heat sensing devices detect the temperature difference between the cells when binding occurs and gives feedback to the heaters for the instrument to keep the two cells at the same temperature. The other binding partner is loaded into a syringe and injected into the sample cell. The molar ratio between the two binding partners gradually increased through a series of injections. Less binding occurs as the reaction reaches saturation and the area of each peak is integrated and plotted versus the molar ratio of ligand to protein to generate the affinity constant. From these measurements the binding mechanism, affinity, and stoichiometry can often be obtained.*

ITC has been thoroughly used to study ligand-macromolecule interactions like RNA-ligand, DNA-ligand, protein-protein and interactions with solid nanoparticle materials. Several recent examples illustrate the scope of the technique. (1) Salim and Feig (2009) used ITC to study the thermodynamics of the assembly of individual subunits in the 30S ribosomal subunit in bacteria and show that S8 and S11 are thermodynamically independent of other proteins, prove S15 had a cooperative effect on the S6/S8 heterodimer, and probe the sum of individual protein binding steps measured in isolation. (2) Velazquez-Campoy *et al.* (2015) used ITC to investigate heterodimeric and homodimeric protein associations of porcine pancreatic trypsin and bovine pancreatic α-

chymotrypsin with soybean trypsin inhibitors. (3) Huang and Lau (2016) used ITC to illuminate the thermodynamic driving forces behind biomolecule-nanoparticle interactions and the effects of the physiochemical properties of both nanoparticles and biomolecules in these interactions. These examples show that ITC is advantageous not only because it is the only technique that can determine all binding parameters in a single experiment and does not require the need of chemical modification, but also because of its robust nature and ability to determine binding affinities in the range from 100 μM to 1 nM. (Callies, 2016) However, ITC can be quite time-consuming and suffers from relatively low sensitivity. This requires larger amounts of material (high sample volumes) than other methods, which often makes it unsuitable for experiments involving determinations on rare or precious samples. (Olmsted, 2012) (Fechner, 2014)

1.2 Surface Plasmon Resonance

Surface Plasmon Resonance (SPR) is an optical method that enables real-time measurement of biomolecular interactions via changes in RI at a sensor surface using a relatively small amount of a binding partner attached to the surface. Some exciting recent examples involving membrane proteins have shown the protein proteins to be displayed in a native or native-like environment. (Patching, 2014) The sensor surface (Figure 2) is usually a uniform thin film of gold (~50 nm) on a glass support that forms the bottom of a <100 nl flow cell. Binding affinity is detected by injecting an aqueous solution containing one binding partner through the flow cell after immobilizing the other binding partner to the sensor surface. (Patching, 2014) SPR relies on total internal reflection of a

monochromatic, *p*-polarized light that travels through an optically dense prism and medium, usually glass, normally coated with the thin gold film. Gold is employed because of its highly stable nature and because it can be readily and densely functionalized by thiol molecules. Surface plasmons are generated at a critical angle dependent upon the refractive index of the prism within 300 nm of the gold surface. Changes to the plasmonic signal are generated as molecules bind to the surface, which are presented as changes in signal intensity to give a sensorgram. (Patching, 2014) (Stahelin, 2013)

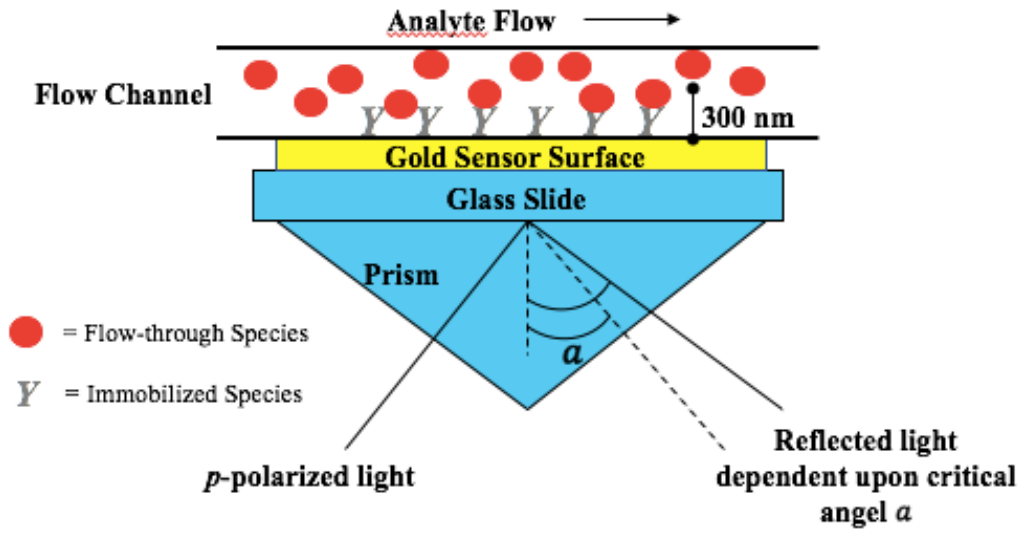

Figure 2. *Typical surface plasmon resonance set-up. One species is immobilized to the gold sensor surface which can be chemically modified to allow attachment of various biomolecules and the other species is injected and flowed over the sensor surface. Binding is detected as the p-polarized light reaches the prism-glass interface and is reflected back and detected by CCD detectors. The critical angel, a, is dependent upon the association at the sensor surface.*

The most widely used SPR-based technology is the Biacore instrument, based on different chemically functionalized surfaces provided in a series of sensor chips that allow the

investigation of various molecular interactions. (GE Healthcare Biacore Sensor Surface Handbook, 2005) Biomolecules can be immobilized on a variety surfaces such as carboxymethylated dextran via amine coupling, gold via interactions with thiol, and streptavidin surfaces via biotin groups. (Citartan, 2013) SPR biosensors have been used to investigate the binding of macromolecules in many bond types, such as DNA-protein, protein-lipid, protein-polysaccharide, cell or virus-protein, etc. The following recent examples are noteworthy. (1) Stockley and Persson (2009) investigated the association between MetJ, the *E.coli* methionine repressor, and a biotin tagged-DNA duplex containing the consensus operator site that was immobilized to a streptavidin surface using the SA sensor chip. (2) Baron and Pauron (2014) studied the binding of an antimicrobial protein to a lipopolysaccharide using a CM5 sensor chip. (3) Kim *et al.* (2017) analyzed the interaction of the Zika Virus envelope protein with glycosaminoglycans using a carboxymethylated dextran streptavidin sensor chip to consider the role of glycosaminoglycans in host cell entry of Zika Virus into placenta and brain.

Similar to ITC, SPR is advantageous because it doesn't require labeling; it surpasses ITC in its ability to monitor interactions and dissociations in real time, high sensitivity, large number of samples that can be performed in a short time, and lower limits of detection. (Olmsted, 2012) (Stahelin, 2013) However, several disadvantages are also evident. First, it can be challenging to obtain reliable and reproducible binding data due to nonspecific binding to the sensor chip, mass transport effects, and protein/lipid surface stability. Second, SPR is sensitive to mass, and so is limited to an interrogation distance of a few hundred nanometers from the surface. Third and most important, it requires one of the

species to be immobilized to the surface which adds chemical complexity to the interaction and makes it potentially fundamentally different from the solution-phase interaction it may be intended to represent. (Olmsted, 2012) (Stahelin, 2013) Thus, a technique which enables the detection and study of binding events in free solution can provide substantial advantages over SPR.

1.3 Backscattering Interferometry

Backscattering interferometry (BSI) is a relatively new free-solution label-free technique that allows the monitoring of binding partners in their native states based on a change in refractive index. (Kussrow, 2011) The change in refractive index occurs when two molecules in solution interact and is sensitive to changes in molecular structure, dipole moment, polarizability, charge distribution, confirmation, and solvation state. (Pesciotta, 2011) BSI (Figure 3) uses a low-powered monochromatic helium-neon (HeNe) laser source to irradiate a microfluidic channel that holds the solution (usually aqueous) containing one of the binding partners. As the laser beam interacts with the aqueous solution, it reflects off of the channel surface producing a high-contrast fringe pattern, the spatial position of which is based on the RI of the fluid in the channel. The interference fringe is directed to a linear charged-coupled device (CCD) detector that monitors changes in the spatial pattern as a function of the addition of the second binding partner to the solution. Fast Fourier transform (FFT) analysis allows the computer to obtain real-time measurements, giving a phase change (in radians) which enables the quantification of the shift by focusing on the

specific frequency of the fringes and calculating the phase information. (Pesciotta, 2011) (Kussrow, 2011) (Olmsted, 2012)

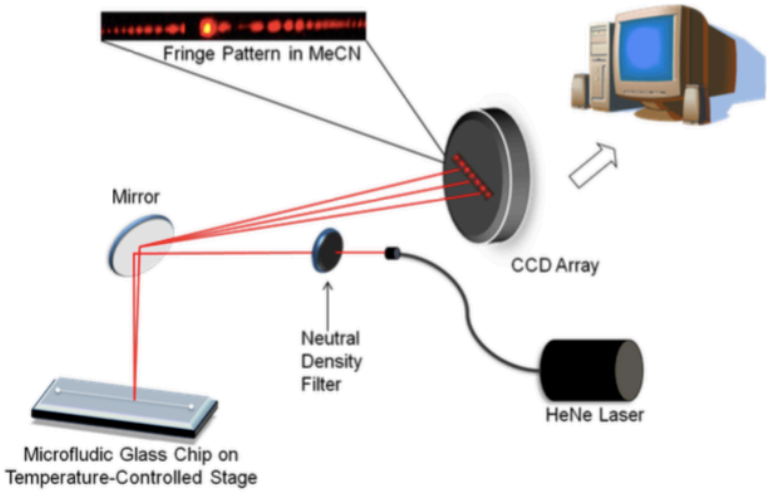

Figure 3. *Schematic of backscattering interferometry instrumentation. Fringe pattern representative of acetonitrile, MeCN. Image reproduced from Pesciotta (2011).*

BSI has been used to explore a variety of molecular interactions and has proven to be a versatile and unique biosensing technique. Baksh *et al.* (2011) used BSI to quantify ligand-receptor binding affinities in a number of membrane proteins in lipid membranes of varying heterogeneity. For example, monosialoganglioside integral membrane GM1-containing vesicles were incubated with varying concentrations of the cholera toxin B subunit. The binding between the membrane-bound molecule and the soluble protein was detected by comparing the observed phase shift to those observed for control mixtures that were identical to the experimental samples except for the presence of GM1 in the membrane preparation. Plots of these properly-referenced phase shifts vs. cholera toxin B concentration resulted in a sigmoidal curve that generated an equilibrium binding constant

of 129 ± 27 pM by Langmuir isotherm analysis, close to the binding constant value obtained previously by other methods. Other reported examples include the binding of fatty acid amide hydrolase (FAAH), CXCR4 receptor, and the $GABA_B$ receptor, all integral membrane proteins, with their various small-molecule ligands or inhibitors.

Other examples have illustrated the high sensitivity of the BSI technique, and its compatibility with a variety of binding interactions. For example, Haddad *et al.* (2012) screened a series of novel acetylcholinesterase (AChE) inhibitors for the advancement of drug discovery efforts for an effective treatment of Alzheimer's disease. The BSI detection limit of 3.6×10^{-5} fmol of AChE per optical probe volume of 360 pL, was found to be exceed the sensitivity of previously used techniques and proved to be well-suited for the rapid screening of dual-binding AChE inhibitors. Nucleic acid aptamers have proven to be especially well-detected by BSI. For example, Adams *et al.* (2013) investigated the use of BSI for biomarker detection by monitoring the interactions of nucleic acid probes with the respiratory syncytial virus (RSV) nucleocapsid (N) gene RNA biomarker. In addition, Kammer *et al.* (2014) demonstrated the capability of BSI to characterize aptamer and small molecule associations by measuring the aptamer affinities of bisphenol A, tenofovir, and epirubicin. The reported values were consistent with those previously determined by other means, but did not require awkward or potentially disruptive labeling operations. These investigators then quantified previously unknown dissociation constants of aptamers to ampicillin, tetracycline, and norepinephrine. Saetear *et al.* (2015) showed BSI could be used to investigate host-parasite protein interactions by quantifying the interaction of two

Plasmodium falciparum invasion ligands, PfEBA175 and PfRH5, with intact and unmodified human erythrocytes.

While BSI is usually used for interactions in free solution, it can also be employed to monitor binding to a species immobilized on the microfluidic channel. (Kussrow, 2011) In both modes of operation, the technique benefits from high sensitivity, optical simplicity, small sample volume requirement (pL to uL range), and broad applicability. (Pesciotta, 2011) (Bornhop, 2016) Because it responds to changes in factors other than mass that are induced by binding, it works equally well for small and large molecules, unlike SPR. (Kussrow, 2011)

CHAPTER 2: NEW ADVANCES IN LABEL-FREE TECHNIQUES

2.1 Bio-layer Interferometry

Bio-layer interferometry (BLI) is an operationally simple dip-and-read system used to measure interactions between proteins, peptides, nucleic acids, small molecules, and lipids in real-time. (Sultana, 2015) The FortéBio system, perhaps the best known commercial implementation of this technique (Figure 4), analyzes the interference pattern of incident white light that is reflected from an internal reference layer and a biomolecular bait that is immobilized using a variety of chemistries on a matrix at the tip of a fiber-optic sensor. (Wallner, 2013) The interaction of two binding partners generates a change in the optical thickness at the tip which results in a wavelength shift proportional to binding. (Sultana, 2015) The requirement for immobilization and the detection of signal in close proximity to the surface are common to BLI and SPR.

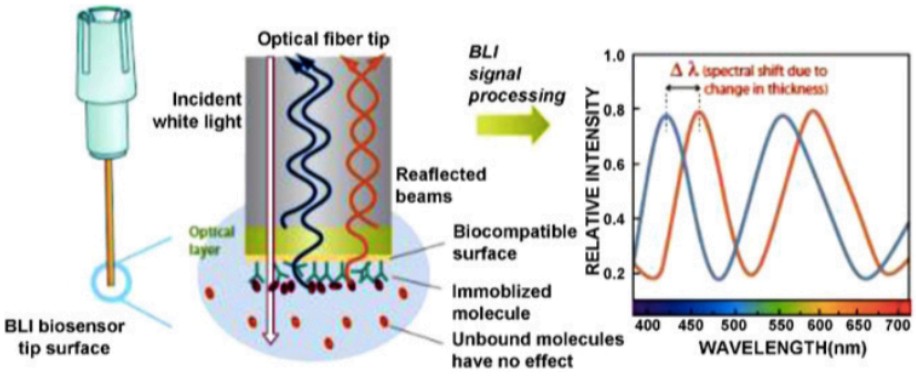

Figure 4. *Operating principle of the bio-layer interferometry technique. A single biosensor-tip is shown with a detailed image of the technique used to analyze the interference pattern of white light. Changes in the number of molecules that bind to the tip results in a shift in the interference pattern indicated by a wavelength shift. Image reproduced from Brandon (2015).*

BLI is useful for studying proteins that are challenging to isolate because it requires only nanomolar amounts of sample. It has the added unique benefits of being a time-efficient technique for studying a large number of samples due to its ability to carry out experiments in parallel. Furthermore, since the bulk of the medium is not sampled during the measurement, BLI can be used to quantify protein interactions from a heterogeneous crude lysate. (Sultana, 2015)

ForteBio produces two biolayer interferometry systems: the BLItz and Octect. The BLItz is a manual, single-channel system that has the advantage of using a droplet of only 4-5 µL of bait or analyte for each kinetics run which allows for a complete set of kinetics data of five concentration points to be obtained from ~25 µL of sample. (Sultana, 2015) The system is most convently applied to interactions between binding partners that are stable at room temperature. For example, Sultana and Lee (2015) used BLItz to analyze several different DNA-protein and protein-protein interactions involved in the role played by APOBEC3F, a key host DNA cystosine deaminase, in the potent restriction of HIV-1 replication.

Although the BLItz has an advantage over the Octet system in experiments where sample amounts are limited, it is limited to analytes larger than 10 kDa with dissociation constants in the mM to nM range. In contrast, the Octect can make measurements at temperatures other than room temperature, and can analyze binding interactions of mM to pM affinity in a multichannel automated format in 96- or 384-well plates. (Sultana, 2015) The system has a variety of modules that can be used to study protein interactions with small molecules

or low molecular weight peptides as low as 150 Da. (Sultana, 2015) Four recent publications highlight the diversity of interactions explored in this way. Brandon *et al.* (2015) demonstrated the ability of the Octet QK system to be used for food analysis applications by characterizing the binding of two monoclonal antibodies, ricin and *Ricinus communis* agglutinin-1(RCA-1). Näreoja *et al.* (2014) used the Octet RED384 model to explore the performance of antibody-functionalized nanoparticles in a sandwich-type immunoassay, a format attractive for diagnostics and screening. Lou *et al.* (2016) studied a representative nucleic acid-protein interaction of a kind beneficial for therapeutics, biosensing, and diagnostics research by observing the binding of an thrombin to an anti-thrombin RNA aptamer using the Octet RED96 instrument. Lastly, Verzijl *et al.* (2017) used the Octet HTX model to monitor the signal transduction in living human A431 epidermoid carcinoma cells in response to drug treatment by exposing them to a variety of small-molecule agonists.

2.2 SPR-based Imaging Technology

SPR-based imaging (SPRi) sensor technology enables simultaneous imaging of ligand surfaces in an array format, essentially multiplexing the regular SPR format. (Yang, 2016) Wasatch Microfluidic's IBIS MX96 model is relatively new to the biosensor field and has shown that it can provide highly reliable and correlated data in comparison with other biosensor platforms. Yang *et al.* (2016) used the IBIS platform to test ten high-affinity mouse-derived monoclonal antibodies for their binding kinetics against the human proprotein convertase substilisin kexin type 9 (PCSK9) antigen in a study that compared

the efficiency of the biosensor platform to three other platforms (Biacore T100, Octet RED384, and ProteOn XPR36). ProteOn XPR36 is another SPR-based technology that offers multiplex options allowing as many as 36 different reactions to be monitored in real-time. (Citartan, 2013) Nahshol *et al.* (2008) showed the rapid screening and selection ability of the ProteOn system by simultaneously screening 250 supernatants against human interleukin 12 (IL-12) and a human hemoglobin variant in ~17 hours.

CHAPTER 3: THE NEWEST LABEL-FREE TECHNOLOGIES

Recently, more label-free technologies have emerged for exploring the affinities and nature of molecular interactions. Each of these technologies can be implemented in micro-scale, label-free systems and have vast potential to satisfy the demand for better quality sensors. Each of these methods requires more research and development before they can take their place among the next generation of biosensor devices.

3.1 Field-Effect Transistor-Based Biosensors

A variety of field-effect transistor-based biosensors (FET-based) have been proposed as having great potential for investigating biomolecular interactions and processes at the nanoscale, including antibody-antigen binding, DNA hybridization, and enzymatic reactions. These technologies are based on one-dimensional semiconducting nanomaterials such as carbon nanotubes (CNT-FETs) and silicon nanowires (SiNW-FETs). In principle, they offer the biosensor the ability to detect molecular interactions in real-time with potential application to large-scale and high-density integration. (Sang, 2015) Two-dimensional semiconducting nanomaterials, such as graphene (GFET), may offer especially high sensitivity and selectivity. (Sang, 2015)

3.2 Magnetoelastic Biosensors

In magnetoelastic (ME) biosensors (Figure 5), the sensor platform is subjected to a time-varying external magnetic field, producing an oscillating magnetic signal which can be detected as it causes changes in the current passing through a pickup coil. (Sang, 2015) Binding events cause very small changes in the magnetic field shape that can be detected in real-time. ME biosensors can therefore be employed in enzyme detection, protein detection, immune reaction, and other applications.

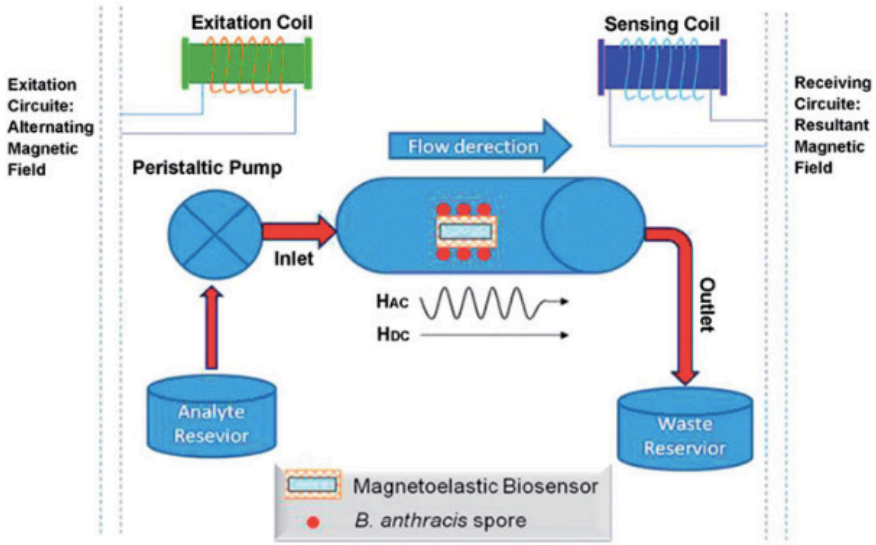

Figure 5. *Schematic of the magnetoelastic biosensor detection system. Image reproduced from Sang (2015).*

3.3 Biophotonic Biosensors

Biophotonic biosensors, is the term given to optical-based systems that use scattering and penetrating light produced by metabolic processes to observe biological systems at

molecular, cellular and organismal levels. (Sang, 2015) This allows some interesting correlations with the structure and function of living cells and organisms, particularly such molecular events as gene expression, protein–protein interaction, and many chemico-physical processes. Because signals are produced by the system undergoing investigation, these methods offer unique opportunities for the early detection of diseases and for new modalities of light-guided and light-activated therapies. (Sang, 2015)

CHAPTER 4: CONCLUSIONS

Label-free technologies provide alternatives for detecting and quantifying biomolecular interactions when standard labels (fluorophores, radiolabels, nanoparticles) are too perturbing, inconvenient, or expensive to use. Here, various prominent label-free techniques have been reviewed with a focus on examples from the past four years. Isothermal titration calorimetry provides direct measurement of thermodynamic binding parameters, but requires large sample volumes which makes it unsuitable for experiments involving precious samples. Surface plasmon resonance can be utilized to investigate many protein associations, but is complicated by the need to attach one binding species to the sensor chip and its mass-weighted response. Backscattering interferometry is a highly versatile and sensitive technique that can explore a variety of ligand-macromolecular interactions, but requires careful referencing and is not yet implemented in high-throughput format. New array-based methods include bio-layer interferometry (BLItz and Octect) based on interacgtions with surface-immobilized species and SPR-based imaging technology (IBIS MX96 and ProteOn XPR36). Lastly, new techniques are on the horizon, such as those enabled by field-effect transistor-based biosensors (CNT-FETs, SiNW-FETs, and GFET), magnetoelastic biosensors, and biophotonic biosensors. Given the large number of variables that define the parameters over which different binding events occur, the available range of analytical techniques to investigate them with the proper sensitivity and selectivity is certain to grow in coming years.

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